

Document Control Number <b>WI-B-T-1-6</b>	<b>WORK INSTRUCTION</b> USDA, APHIS, PPQ, CPHST, National Plant Germplasm and Quarantine Laboratory, Bldg 580, BARC-East, Beltsville, MD 20705	Revision Number <b>6</b>
Effective Date: <b>June 4, 2007</b>	Quantitative Multiplex Real-Time PCR (qPCR) for Detection of <i>Phytophthora ramorum</i> Using a TaqMan System on the Cepheid SmartCycler® and the ABI 7900/7000	<b>Page 1 of 14</b>

The purpose of this work instruction is to describe all activities concerning the Real-Time PCR (qPCR) detection of *Phytophthora ramorum* using a TaqMan system on the Cepheid Smart Cyclyer ® II, ABI 7900 or ABI 7000 thermocyclers.

### **Introduction**

The quantitative PCR (qPCR) described was originally developed by the Central Science Laboratory (CSL), York, UK and was validated by USDA-APHIS-PPQ-CPHST to provide more rapid diagnostic determinations for the *Phytophthora ramorum* emergency program. Minor modifications were made for optimization of the assay on a Cepheid SmartCycler® II, including a few chemical and physical parameter changes; however, the primers and probe for *P. ramorum* and the COX internal control primers and probe are those provided in the CSL method.

The test utilizes internal control primers and probe based on the COX gene present in plants that are multiplexed (simultaneously amplified) with *P. ramorum* primers and probe for each DNA sample. The COX internal control adds value to the assay by confirming that amplifiable plant DNA is present in environmental samples (i.e.: leaves containing *P. ramorum*) that test PCR-negative for *P. ramorum* by verifying that an efficient nucleic acid extraction occurred. The qPCR protocol can also be used for on-site detection of *P. ramorum* in the Cepheid system. *Note: Cepheid Omnimix® or SmartMix beads are not to be used in this protocol.*

This work instruction uses purified DNA, isolated from plant samples using the Qiagen DNeasy® Plant Mini Kit, and can detect *P. ramorum* in a broad range of host plants. The range of detection (the interval between the upper and the lower concentration of the pathogen in environmental samples from which the agent can be reliably and reproducibly detected) of this protocol is from 200 ng to 2 fg of genomic DNA. To date, DNA tested with this protocol has shown little to no interference due to plant inhibitors.

### **International Inter-laboratory Validation (Ring-Test) of this Protocol**

The protocol listed in this work instruction was evaluated in an international inter-laboratory validation involving seven laboratories with consistent results.

High concentration genomic DNAs were generated when DNA was isolated from cultures of *Phytophthora* species. Highly concentrated DNA of *P. foliorum* and *P. lateralis* exhibited cross amplification in these tests. Environmental DNA samples of *P. foliorum* and *P. lateralis* that, by the nature of the sample, contain a lower percentage of specific

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target DNA showed late cycle cross amplification with crossing thresholds (Cts) of 38 and higher, and only a few Ct readings of approximately 35.

During the inter-lab validation using the Cepheid SmartCycler® platform, some of the participating laboratories were asked to extend the protocol to two models of the ABI system platforms (models 7900 and 7000) and successful results were achieved eliminating the cross reactivity of the assay. Included in this work instruction are the parameters for the ABI 7900 that were determined by the USDA-AMS Laboratory in Gastonia, NC and were additionally verified successfully on the ABI 7000 by the University of Tennessee. (Information for reagents or PCR parameters specific to the ABI platforms is presented in blue font.)

## **A. Materials and Equipment:**

### **Equipment needed:**

PCR work station

Cepheid SmartCycler® II (version 2), or ABI 7900 or ABI 7000

Cepheid mini-centrifuge (Cepheid # 900-0020)

Vortex

Micro-centrifuge, bench top, capable of  $\geq 10,000$  rpm

Dedicated, calibrated pipettors (P10, P50, P200)

### **Reagents and Materials needed for Cepheid platform:**

SmartCycler® reaction tubes, 25  $\mu$ l (Cepheid #900-0022)

Cepheid cooling block (Cepheid #900-0028)

Molecular Biology Grade (MG) Water (i.e: Eppendorf #955-15-503-3)

Platinum *Taq* DNA Polymerase (Invitrogen #10966-034,)

dNTP Mix (10 mM solution, PCR reagent) (Sigma # D7295)

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D-(+)-Trehalose dehydrate (Sigma #T9531-10G)

Sterile filter (barrier) pipette tips (P10, P50, P200)

Amber-colored 1.5 ml Flex tubes (Eppendorf # 22-36-351-4,)

Micro-centrifuge tubes, 1.5-1.7 ml, (pre-sterilized, certified DNase & RNase free, any vendor)

Tube openers (any vendor)

**NOTE:** All flip-cap microtubes are to be opened using a decontaminated tube opener. Tube openers are decontaminated by soaking 2 hours in a 10% bleach solution, followed by two rinses in water and diH<sub>2</sub>O to remove bleach residue.

**Reagents and Materials needed for ABI platform:** Part number information for ABI TaqMan Master Mix and PCR plates is available from your ABI representative.

#### TaqMan primers

Primer Name	Primer Sequence (synthesized by Integrated DNA Technologies, Inc.) (Purification - Standard Desalting)
Pram-114F	5' – TCA TGG CGA GCG CTT GA -3'
Pram-1527-190R	5' – AGT ATA TTC AGT ATT TAG GAA TGG GTT TAA AAA GT – 3'
COX-F	5' – CGT CGC ATT CCA GAT TAT CCA – 3'
COX-RW	5' – CAA CTA CGG ATA TAT AAG RRC CRR AAC TG – 3'

**TaqMan probes-** Note that label for the COX- Probe varies depending on the machine.

Probe	Probe sequence for Cepheid (synthesized by Integrated DNA Technologies, Inc.) (Purification - Dual HPLC purification)
Pram1527-134T	5' - /56-FAM/TTC GGG TCT GAG CTA GTA G/3BHQ_1/ - 3'
COX-P	5' - /5TexRd-XN/AGG GCA TTC CAT CCA GCG TAA GCA /3BHQ_2/ - 3'
	<b>Probe sequence for ABI 7900 or ABI 7000</b>
Pram1527-134T	5' - /56-FAM/TTC GGG TCT GAG CTA GTA G/3BHQ_1/ - 3'
COX-P	5' - /5VIC-XN/AGG GCA TTC CAT CCA GCG TAA GCA /3BHQ_2/ - 3'

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#### **A. Reagent Preparation**

**Note:** Reagent preparation should be completed once, prior to conducting the PCR assay, generally upon receipt of new reagents. Reagent solutions are stored in small aliquots in a -20°C freezer until needed. When an assay is being conducted, the analyst should use a ‘working’ tube of primers and probe, and a tube of Trehalose.

- Freezer stock solutions (100 µM ) of the probes and primers  
Dissolve primers and probes in MG water (or 1x TE buffer) for a final concentration of 100 µM and store at -20°C. Store freezer stocks of probes in amber-colored microcentrifuge tubes.
- Working solutions of primer mixes  
Prepare 2 µM working solutions of each of the two PCR primer pair mixtures one for the Pram primer pair (Pram114F/Pram1527-190R) and one for the COX primer pair (COX-F/RW). Once diluted, the final concentration of each primer in the mix is 2 µM. Store 100 µl aliquots at -20°C  
For example, to make 1 ml of a 2 µM mix of primers Pram114F/Pram1527-190R, add 20 µl of the 100 µM Pram114 primer stock and 20 µl of the 100 µM Pram1527-190R primer stock to a 1.5 ml microfuge tube and bring up to 1 ml with 960 µl of sterile molecular grade water. (Do the same for the COX-F/RW primer pair.)  
Note: The ABI primer working solutions are *not* prepared as mixes.
- Working solutions of probes  
Prepare 2 µM solutions of each probe in molecular biology grade H<sub>2</sub>O or 1x TE buffer by diluting the 100 µM probe stock solution. Store small aliquots (i.e.: 20 µl) of probes at -20°C in 1.5 ml amber-colored microfuge tubes to protect the primer from degradation due to light exposure. Small aliquots, suitable for one-day use, of probe are desirable since probes are sensitive to frequent freeze/thaw cycles.
- Trehalose  
For Cepheid platform: Prepare a 25% solution of Trehalose: 2.5 g Trehalose is brought up to 10 ml with MG water, stirred to dissolve, and 1 ml aliquots are stored at -20°C.

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For ABI platform: Prepare a 50% solution of Trehalose: 5.0 g Trehalose is brought up to 10 ml with MG water, stirred to dissolve, and 1 ml aliquots are stored at -20°C. The concentration is doubled, compared to the Cepheid platform, due to volume constraints of the ABI PCR master mix.

### **B. Sample Preparation**

Thaw frozen DNA samples briefly (several minutes) then vortex 5-10 seconds at speed setting of 7-10. Briefly spin the tubes 10-20 seconds at  $\geq 10,000$  rpm in a bench top microcentrifuge to settle the liquid at the bottom of the tube. Place in ice.

Samples are tested undiluted and 1:10 so prepare the 1:10 sample dilutions using MG water. Vortex 5-10 seconds at a speed setting of 7-10, and spin the tubes 10-20 seconds at  $\geq 10,000$  rpm in a bench top microcentrifuge to settle the liquid at the bottom of the tube. Place in ice.

Thaw the primers, probe, and all other reagents *except Taq*. Vortex for 10-15 sec at setting 7-10, then centrifuge 10-20 seconds at  $\geq 10,000$  rpm to settle the liquid to the bottom of the tube. Place in ice. The Taq Polymerase should be kept on ice all the time. We do not recommend vortexing the enzyme but gently mixing it by flicking the tube.

Label SmartCycler® tubes.

Remove the Cepheid cooling block from the 4 °C refrigerator and place in ice.

### **C. Master Mix Preparation**

Reagents used in the master mix depend on the platform/thermocycler being used. Below are two mixes, one for the Cepheid SmartCycler (Table 1) and ABI 7900/ ABI 7000 (Table 2).

Prepare the master mix inside a clean PCR workstation and make the mix volume for the number of the samples plus additional (1 for every 10) samples, plus 1 positive (DNA) control and 1 negative/non-template (H<sub>2</sub>O) control. Keep master mix on ice once prepared and prior to aliquoting for individual reactions.

#### **C1. Master Mix Preparation for Cepheid SmartCycler®:**

Prepare the standard master mix as described in Table 1.

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**Table 1: Master Mix for the Identification of *P. ramorum* using the Cepheid SmartCycler®.**

Reagents	Volume (µl) for each reaction	Volume (µl) 16 reactions	Volume (µl) 17 reactions (16 + 1 rxn)*	Final concentration
10X PCR Buffer**	2.5	40.0	42.5	1X
H <sub>2</sub> O (sterile molecular grade)	2.7	43.2	45.9	
Trehalose (25%)	5.0	80.0	85.0	5%
MgCl <sub>2</sub> (50 mM)	3.0	48.0	51.0	6.0 mM
Primers Pram 114F/1527- 190R (2 µM mix)	3.0	48.0	51.0	240 nM
Probe Pram 1527-134-T (2 µM)	1.5	24.0	25.5	120 nM
Primers COX-F/RW (2 µM mix)	3.0	48.0	51.0	240 nM
Probe COX (2 µM)	1.5	24.0	25.5	120 nM
dNTPs (10 mM each)	0.6	9.6	10.2	240 µM
Platinum Taq (5U/µl)	0.2	3.2	3.4	1 U
Total	23.0	368.0	391.0	N/A

\* Use this formula to ensure enough volume is made for all 16 reactions.

\*\*The 10X buffer and MgCl<sub>2</sub> are supplied with the Platinum Taq.

Mix Cepheid Master Mix by pipetting up and down several times, then aliquot 23.0 µl into each Cepheid 25 µl tube, and place tubes in an ice-cold Cepheid cooling block.

## **C2. Master Mix Preparation for the ABI 7900 / ABI 7000:**

Prepare the standard master mix as described in Table 2.

**Table 2: Standard Master Mix for qPCR for the Identification of *P. ramorum* using the ABI 7900 / ABI 7000.**

Reagents	Volume (µl) for each reaction	For 100* reactions (96+4) (µl)	Final concentration
2X Taqman Master Mix (w/UNG)	12.5	1,250.0	1X
Pram Primer 114F (2 µM )	0.625 **	62.5	50 nM
Pram Primer 1527-190R (2 µM)	2.5	250.0	200 nM
Probe Pram 1527-134-T (2 µM)	1.25	125.0	100 nM
Primer COX-F (2 µM)	1.25	125.0	100 nM
Primer COX-RW (2 µM)	1.25	125.0	100 nM
Probe COX-P (2 µM)	1.25	125.0	100nM
Trehalose (50%)	2.375	237.50	5%
Total	23.0	2,300.0	

\*Four additional reactions were added to have extra master mix reagents so there is adequate volume for the 96 PCR reactions.

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**\*\*Please note that the asymmetric primer (Pram 114F) concentration is correct.**

Mix Master Mix by pipetting up and down several times.

Pipette 23.0 µl of Master Mix into each of the 96-wells in the ABI PCR plate.

#### **D. Addition of Sample or Control DNAs**

Each diagnostic run of the *P. ramorum* qPCR must include one *P. ramorum* positive control and one negative control (negative template control containing either TE buffer or H<sub>2</sub>O). Whenever possible, include an environmental sample known to be positive for *P. ramorum* when testing environmental samples.

Note: Do not use 25 µl of master mix as your NTC. The negative control is used to indicate contamination of master mix reagents or contamination introduced by the analyst during sample addition so it should be prepared in a similar manner including the addition of 2 µl of MG water instead of DNA.

Note: If the positive control is *P. ramorum* genomic DNA, a dilution series and qPCR will need to be performed prior to sample testing to determine a working dilution/concentration for subsequent PCR assays.

Addition of samples and controls should be accomplished in the thermocycler room, not in the PCR workstation. The total reaction volume is 25 µl

- Add 2 µl of each sample DNA (undiluted and 1:10 dilution) to the corresponding 25 µl Cepheid tubes (Cepheid platform) or well ([ABI platform](#)). Close the tubes.
- Add 2 µl of each control to the corresponding 25 µl Cepheid tubes (Cepheid platform) or well ([ABI platform](#)). Close the tubes.

#### **E. Verification of cycler settings**

##### **E1. Cepheid SmartCycler® Set-up:**

Verify the following settings on the SmartCycler® II prior to use:

- Stage 1: Hold at 95°C for 20 seconds with optics off.
- Stage 2: repeat 46 times and 2-Temperature Cycle.

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- the first temperature cycle, set 95°C for 1 second with optics off
- the second temperature cycle, set 58°C for 40 seconds with optics on.

Use a temperature ramping rate of 3.0 degrees per second.

## **E2. ABI 7900 / ABI 7000 System Settings:**

Verify the following setting on the ABI 7900 / ABI 7000 systems:

- a. Stage 1: Hold at 50 °C for 2 minutes
- b. Stage 2: Hold at 95 °C for 10 minutes
- c. Stage 3: Repeat 46 times
  1. First Temperature Cycle, 95 °C for 15 seconds
  2. Second Temperature Cycle, 58 °C for 15 seconds
  3. Third Temperature Cycle, 72 °C for 30 seconds

Use a ramping rate of 3.5 degrees per second.

**Note:** To reduce the potential of false negative results the *P. ramorum* qPCR method cycle length parameter was adjusted to 46 cycles. The validated *P. ramorum* qPCR method was compared to the existing nested *P. ramorum* PCR procedure on over 800 environmental DNA samples during the second phase of the validation procedure. Analysis of this data indicates that there are a very small percentage of environmental DNA samples with Ct values between 40 and 46 that test positive in the nested PCR procedure.

## **F. Starting the run**

Cepheid Smart Cycler:

1. **Turn on the SmartCycler® Block first, followed by the SmartCycler® software. If not done in this order the machine will give an error message.**
2. Spin the Cepheid tubes in the Cepheid centrifuge for 10 seconds. Make sure no bubbles remain on the sides of the flat diamond-shaped area of the Cepheid tube. Place the tubes in the SmartCycler® in the appropriate I-core modules.
3. To begin amplification: (instructions contained in the SmartCycler® manual)
  - i. Select "Create Run"
  - ii. Give the run a name (i.e.: date and protocol)
  - iii. Select dye set (for this qPCR it is "FTTC25")
  - iv. Select "add/Remove Sites" to select the protocol

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- v. Select the sites with the samples and click on ► symbol to add sites to the right column, and click on OKAY.
- vi. Select “Start Run” found in the lower left-hand corner of the screen.  
(Steps 2-4i. through v. can be completed prior to master mix set-up).

ABI platform: Consult manual.

#### **G. Assessment of the *P. ramorum* qPCR using the SmartCycler® software Version 2**

Analyze data from the run as per the thermocycler Manufacturer’s instructions.

For the sake of discussion in sections F.1 to F.3 assume that FAM is the *P. ramorum* probe fluorescent label and that Texas Red (TxR) is the COX internal control probe fluorescent label.

(This is an example for the Cepheid SmartCycler® system so insert the VIC-labeled COX control in this explanation if an ABI platform is used.)

#### **F1. Assessment of COX Internal Control (TxR dye) Cts:**

The COX primers target plant DNA sequences only and will not amplify *Phytophthora* or *P. ramorum* genomic DNA, including positive controls from culture (genomic) DNA. The COX internal control reaction in an environmental sample will typically exhibit Cts in the low 20s to middle 30s when amplifiable DNA is present. A positive result in the COX assay verifies the degree to which the DNA extraction was successful and provides evidence that *P. ramorum* samples testing negative are due to the lack of *P. ramorum* target or levels of *P. ramorum* target below the limit of detection of the assay, and not due to poor DNA extraction.

If an environmental positive control has a COX internal control Ct = 0, or very low Ct ≤ 13, or very high Ct = 36-46, then the entire run is invalid. No sample results obtained in the run may be used and all samples must be retested.

**If an environmental sample has a COX internal control Ct = 0, or very low Ct ≤ 13, or very high Ct = 36-46, then that specific sample needs to be retested.**

NOTE: Observations made by NPGBL and some field laboratories indicate that some environmental samples positive for *P. ramorum* may test negative for the

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COX internal control (no TxR Ct). In most cases the FAM Ct for *P. ramorum* is low, indicating high target DNA concentration that can inhibit the internal control amplification. The internal control amplification is more important in cases when sample is negative for *P. ramorum* since in this case a positive COX reaction indicates that sample is negative for *P. ramorum* because of the absence of the pathogen target not because of bad quality DNA or presence of inhibitors.

To resolve the problem three courses of action can be followed:

- 1) Dilute the DNA further (1:100 to 1:1,000) and repeat the real-time PCR; however, this may not work since inhibition sometimes depends on the targets ratio and not absolute concentration. If the retest again produces no fluorescence (COX internal control Ct = 0-13) or very high fluorescence (Ct = 36-46) then the sample has to be re-extracted and retested;
- 2) Rerun a 1:10 and 1:100 dilution of the sample in the *P. ramorum* conventional Multiplex PCR assay. The internal control amplification in the conventional Multiplex PCR is less likely to be inhibited by high *P. ramorum* concentration;
- 3) Rerun the real-time assay for the sample(s) in question as a simplex PCR with the COX primers and probe only.

## **F2. Assessment of positive and negative control (FAM dye) Cts:**

If the positive control DNA (environmental or pure culture DNA) tests negative (i.e.: FAM Ct = 0 or very low Ct  $\leq$  13, or very high Ct 36-46), or your negative (NTC) control (H<sub>2</sub>O or TE buffer only) tests positive (i.e.: FAM Ct > 0) the entire run is invalid. No sample results may be used and all samples must be retested.

Positive controls from the same genomic DNA isolation (from *P. ramorum* culture) should give a similar FAM Ct when used at the same dilution factor in subsequent PCR reactions. For example, a 1:100 dilution used at 2  $\mu$ l in runs 1, 5, and 10 should have FAM Cts that are close,  $\pm$  1 to 3 Cts.

A positive control (environmental sample or genomic DNA) that tests negative should be retested at a dilution of 1:100 to determine if the PCR was inhibited because the positive control DNA was highly concentrated.

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### **F3. Assessment of Sample (FAM dye) Cts:**

Samples are only determined to be positive, negative or inconclusive for *P. ramorum*. (See COX section for interpreting sample COX Ct values)

#### **Positive samples:**

A sample is determined to test positive for *P. ramorum* if it produces a FAM Ct value between 14 and 36.00 and the controls (both those for the run and for the COX internal controls for individual reactions) gave the expected results, and no Ct was present in the negative control reaction.

Samples which test positive for *P. ramorum* should be retested by qPCR to confirm the first run results.

*Note: See Section F1 for resolving samples that are FAM positive but COX negative.*

#### **Inconclusive samples:**

Samples that have out-of-range FAM or Texas Red Ct's (36 to 46) should be diluted at 1:100 and retested in qPCR.

#### **After the qPCR retest:**

- if the COX Ct's are 0, the sample is determined to be inconclusive and a new sample should be requested.
- If Ct values are again in the range of 36-46 then the DNA sample must be subjected to evaluation using the validated *P. ramorum* conventional multiplex PCR.\*

*An inconclusive result should be determined if the recommended retest and dilution do not produce a result in the assay that can be determined as either positive or negative.*

#### **Negative samples:**

Samples that have FAM Cts of 0.00 are considered negative.

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Effective Date: <b>June 4, 2007</b>	Quantitative Multiplex Real-Time PCR (qPCR) for Detection of <i>Phytophthora ramorum</i> Using a TaqMan System on the Cepheid SmartCycler® and the ABI 7900/7000	<b>Page 12 of 14</b>

*\* If there are doubts about any sample with Cts between 36 and 46, or about results from the follow-up nested PCR evaluation, the DNA sample can either be re-extracted and re-tested, or be forwarded to an approved laboratory for further analysis. Note: If this is a **Potentially Actionable Suspect Sample (PASS)** in need of federal confirmation, see submission instructions below.*

#### **G. Samples for Federal confirmation:**

A **Potentially Actionable Suspect Sample (PASS)** is a sample from a potential new location, a putative new host, a previously certified nursery, a geographically distinct/unique environmental find, or other non-routine situation. For more information, [http://www.aphis.usda.gov/plant\\_health/plant\\_pest\\_info/pram/downloads/pdf\\_files/passpol icy4-06.pdf](http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/pdf_files/passpol icy4-06.pdf)

A **PASS sample** must be forwarded to a federal lab for confirmation (see submission information below).

Samples are submitted by overnight delivery and must be accompanied by (1) a PPQ Form 391 (Sample Submission Form), (2) labeled gel photos and/or Ct values for each run (see example table below).

**Prior to sending material** please notify Dr. Palm by e-mail (Mary.Palm@aphis.usda.gov) with the tracking number and estimated delivery date using the shipping address below:

USDA-APHIS-PPQ-PHP-MDL  
BARC-East, Bldg 580  
Powder Mill Rd  
Beltsville, MD 20705  
Phone 301-504-5700

**Example of Table of qPCR data to accompany the PPQ Form 391 and sample submitted to NPGBL**

Sample ID	PCR Run #	Dilution	P. ram FAM Ct Value	COX TxR Ct Value
SOD-MD-0113	1	Und	36.5	24.0
	1	1:10	36.9	26.8
	2	1:100	37.9	28.5

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### **Definitions:**

**Environmental Sample:** DNA isolated through a nucleic extraction process from plant material.

**Genomic DNA:** DNA isolated through a nucleic extraction process from pure *Phytophthora ramorum* culture.

**Potentially Actionable Suspect Sample:** A PASS sample is a sample from a new location, from a putative new host, previously certified nursery, unique environmental find, or other non-routine situation.

### **References:**

1. K.J.D. Hughes, Central Science Laboratory, SOP No. PLHB/M28: Diagnosis of *Phytophthora ramorum* by TaqMan PCR. Version dated 09-28-2003.
2. J. Tomlinson, Central Science Laboratory, SOP No. PLHB/M31: Use of the Cepheid Smart Cycler II for on-site detection of *Phytophthora ramorum*. Version dated 04-23-2004.
3. SmartCycler® II Operator Manual Copyright 1999-2002 by Cepheid.

### **Document Revision History**

Status (Original/Revision/C ancelled)	Document Revision Number	Effective Date	Description
Original	Original	06/20/2005	Quantitative real-time PCR qPCR for <i>P. ramorum</i>
Revision	1	10/09/2005	Added additional language to the introduction, added further explanation to the test evaluation. Added an example of a data table that must accompany PASS samples for testing at NPGBL. Added definitions and references.
Revision	2	10/24/2005	Added additional information to the introduction regarding the interlab validation test data, included the chemical and physical parameters of the ABI 7900 / ABI 7000 instruments as a result of the ring-test. Data in ring-test regarding BioGene Hot Taq that reduced cross reactions in the Cepheid system were not added to the WI needing further investigation and because the enzyme is not available in the US. Added definition of the PASS sample. Reformatted.

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Revision	3	2/14/2006	Added comment to section A2 that OmniMix beads are not to be used with this protocol. Removed reference to WI-B-T-0-5 and WI-B-T-0-6 in section B2. Corrected Table 1 for dNTPs to read 10 mM each. Corrected Table 2 to remove ‘# of beads’ from volume column heading. Clarified PASS in sections C2 and C3. Changed ‘should’ to ‘must’ in section D and reworded definition.
Revision	4	4/03/2006	Added language to clarify resolution of non-conforming results for the internal control (COX) assay (page 8, C.2)
Revision	5	2/23/2007	Added language to clarify preparation of reagents and correctness of asymmetric primer in ABI master mix. Added probe info for ABI platforms. Added third choice for resolving lack of COX amplification. Corrected Trehalose concentration for ABI platform. Corrected units of dNTP final concentration. Clarified Inconclusive sample retesting. Added requirements for using a PCR workstation when preparing master mix and use of tube openers. Replaced term ‘ring test’ with ‘interlab validation’. Clarified the 3 terms for sample FAM results permitted. Added APHIS-PPQ website link for PASS system.
Revision	6	6/04/2007	Removed requirement for using a 0.6 ml tube prior to loading the Cepheid tube. Added use of cooling block. Updated PASS website location. Updated federal lab (confirmation) information to be MDL. Removed gel loading tips from material list. Changed microcentrifuge tube size on materials list from 0.6 to 1.5-1.7 ml.

**Approved: Signature on File with Original Document**

Date: 6-04-2007

Approved By: Renee M. DeVries, NPGBL Quality Manager

Approved By: Laurene Levy, NPGBL Technical Manager

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